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14. ABSTRACT:
Kaiso is a novel member of the POZ-ZF family of transcription factors, first identified as a binding partner to the p120ctn cell adhesion co-factor. Preliminary work in our laboratory indicated that Kaiso was misexpressed in ~40% of human breast tumors and identified cyclin D1 as a putative Kaiso target gene. The objective of this project is to determine the mechanism by which Kaiso regulates cyclin D1 expression and how this affects breast cell proliferation and tumorigenesis. Using techniques such as artificial promoter assays, electrophoretic mobility shift assays and semi-quantitative PCR we sought to determine how Kaiso regulates cyclin D1 promoter binding and expression. Our work to date demonstrates that Kaiso transcriptionally represses the cyclin D1 promoter through it's bimodal binding properties (binding to both sequence specific sites and methylated CpG sites within the promoter). We have also demonstrated that Kaiso and p120ctn activity may be modulating canonical Wnt signaling and activation of cyclin D1.

15. SUBJECT TERMS
Kaiso, p120ctn, POZ-ZF, transcription factor, breast tumorigenesis, target gene, cyclin D1

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INTRODUCTION

The majority of fatal and invasive human tumors derive from epithelial origin. They are characterized by their reduced intercellular adhesion, and increased mobility to secondary sites [1]. One complex that is essential to epithelial cell adhesion stability and function is the E-cadherin-catenin complex. Malfunction of this complex has been implicated as a contributing factor to ~50% of metastatic human carcinomas [1, 2]. E-cadherin is a transmembrane cell adhesion molecule that facilitates cell-cell adhesion via calcium-dependent homophilic interactions with other E-cadherin molecules on adjacent cells [3]. In the cytosol E-cadherin is anchored to the actin cytoskeleton via its interactions with the catenin proteins (α-, β-, γ-, and $p120^{ctn}$). β-catenin binds to the catenin binding domain (CBD) of E-cadherin and links E-cadherin to the actin cytoskeleton via α-catenin [4]. E-cadherin also interacts with $p120^{ctn}$ (hereafter p120) at its juxtamembrane domain (JMD) [5]. Although there is a correlation between tumor invasion, metastatic characteristics and the integrity of the E-cadherin/catenin complex, some tumors still retain normal expression of E-cadherin and the classical catenins (β- and γ-). This implicates other catenins, for example p120, as important factors in tumor progression. A yeast-two hybrid screen using p120 as bait was performed by my supervisor, Dr. Juliet Daniel, during her post-doctoral tenure to identify potential p120 binding partners. The novel POZ-zinc finger (ZF) transcription factor, Kaiso was identified [6]. Kaiso is a dual specificity transcription factor that is misexpressed in ~40% of human breast tumors. This is consistent with the fact that POZ-ZF transcription factors have been implicated in cancer as both oncoproteins and tumor suppressors [6-8]. Kaiso is also a potential regulator of the cell cycle regulatory gene *cyclin D1*, which is known to be aberrantly expressed in human breast cancers [9-11]. Our initial studies (based on the research performed by the previous P.I. for this award, Ms. Abena Otchere) revealed that Kaiso binds the *cyclin D1* promoter region *in vitro* and *in vivo* and represses the *cyclin D1* promoter. We also found that p120 activates a minimal *cyclin D1*-promoter and that Kaiso inhibits β-catenin-mediated activation of the minimal *cyclin D1*-promoter. These initial findings suggested that Kaiso and p120 may be involved in a signaling pathway, where Kaiso regulates genes involved in tumorigenesis and its binding partner p120 serves to modulate its function. This possibility led to our hypothesis that **Kaiso plays a role in breast cell proliferation and tumorigenesis via its regulation of *cyclin D1* expression**.

Ms. Abena Otchere, the former P.I for this grant, graduated and entered medical school approximately one year after the award was granted to her. The grant was subsequently awarded to me in September 2006 when I began my graduate studies with Dr. Daniel. Below is a summary of my progress in the past seven months.

BODY - PROGRESS

Specific Aim 1: Examine Kaiso expression and function in human breast tumors.

Aim 1.1: Establish a correlation between Kaiso and Cyclin D1 expression in breast tumors. I am currently optimizing our Kaiso-specific monoclonal antibodies for use in immunohistochemistry studies and tissue microarray. Analysis of 650 annotated breast tumor tissue samples will then be performed in collaboration with Dr. David Rimm at the Yale University Cancer Center using automated quantitative analysis (AQUA), an objective method of scoring developed by Dr. Rimm's laboratory.

Aim 1.2: Generate a Kaiso-overexpression model system. In order to create a human breast cancer Kaiso-overexpression cell line, MCF7 breast tumor cells were transfected with the pcDNA3-hKaiso expression construct. The pcDNA3 plasmid contains a neomycin resistance gene which is used as a selectable marker for stable cell line creation (Invitrogen). I have successfully completed the selection stage of this process and I am currently in the final cell expansion stage of this process. Next, I will be testing putative colonies for Kaiso overexpression. Kaiso-overexpressing cell lines will then be used along with other Kaiso misexpression cell lines created within our laboratory, to complete **Specific Aim 1.3** which is to determine the effects of Kaiso misexpression on breast cell growth and transformation.

Outcome:

1. **Optimization of Kaiso-specific monoclonal antibodies for immunohistochemistry.**
2. **Generation of a Kaiso overexpression breast tumor stable cell line.**

Specific Aim 2: Characterize cyclin D1 as a putative Kaiso target gene.

Aim 2.1: Determine if Kaiso interacts with the *cyclinD1* promoter in a methylation-dependent manner. Two Kaiso binding sites (KBS), located -1118 and +24 in relation to the transcriptional start site within the *cyclin D1* promoter, had previously been tested for Kaiso-specific binding by the former P.I. Ms. Abena Otchere. A third KBS (+1050) was subsequently found within the first intron of *cyclin D1*. An oligonucleotide spanning this region was created to be used in electrophoretic mobility shift assays (EMSAs) to test for Kaiso binding. Unlike the other two KBS sites (-1118 and +24), Kaiso does not bind this +1050 KBS region in either a methylation-dependent or independent manner **(Figure 1, Appendix)**.

During our characterization of the *cyclin D1* promoter, it became apparent that Kaiso may be repressing the *cyclin D1* promoter in a methylation-dependent manner. This led us to search the *cyclin D1* promoter region for CpG islands and we identified 10 potential CpG-dinucleotide pairs (i.e. CpGCpG) that may be recognized and bound by Kaiso. Eight oligonucleotides were created to span each of these sequences. These oligonucleotides were tested for their ability to bind Kaiso in both a methylation-dependent and independent manner in EMSA experiments. It was found that Kaiso bound all eight of the methylated but not unmethylated oligonucleotides **(Figure 2)**. However it was apparent that although Kaiso was binding to all of the methylated CpGCpG sequences, it did so with different affinities. To determine the relative affinity of Kaiso binding to the eight CpG-site oligonucleotides, an EMSA using all eight probes with the GST-hKaiso ZF construct was performed. Kaiso bound CpG5 and CpG8 oligonucleotides with higher affinity than any other CpG site, suggesting that these sites were more physiologically relevant **(Figure 3)**. These two strong binding sites (CpG5 & CpG8) and the two weakest binding sites (CpG3 & CpG6) were further tested for binding to the full-length wild-type Kaiso and Kaiso-zinc finger point mutant. It was noticed that although the two strongest binding CpG sites bound to the full-length Kaiso-GST fusion protein **(Figure 4A)** the two weakest sites appeared not to bind to full-length Kaiso **(Figure 4B)**. This again suggested that the two strongest binding CpG sites, CpG5 and CpG8, represent more physiologically relevant sites.

To test the effect of Kaiso-mediated methylation-specific repression of the *cyclin D1* promoter, a series of promoter-reporter luciferase assays were performed following *in vitro* methylation of the 1748CD1 *cyclin D1* partial promoter-reporter construct. We found that when methylated, the 1748CD1 construct was fully repressed both in the presence and absence of ectopic Kaiso **(Figure 5)**. However, the possibility remained that the effects observed were due to endogenous Kaiso. To address this

possibility I am currently performing Kaiso knockdown expression studies using siRNA and testing the effect that endogenous Kaiso has on methylation-dependent repression of the 1748CD1 construct.

To test the effects that loss of methylation might have on Kaiso-mediated repression of the *cyclin D1* promoter, a series of luciferase assays were performed using 5'azacytidine (a methyltransferase inhibitor) treatment. Cells were treated with 2.0μM 5'azacytidine (5'aza) for 48 hours and then co-transfected the 1748CD1 partial *cyclin D1* promoter reporter construct with Kaiso. We observed a slight decrease in expression of the *cyclin D1* promoter following 5'azacytidine treatment both in the presence and absence of ectopic Kaiso **(Figure 6)**. These results were unexpected and may represent the effect of partial methylation of the *cyclin D1* promoter. Further studies need to be performed using higher concentrations of 5'azacytidine to ensure that all CpG sites are de-methylated.

As a result of the above findings, it became necessary to determine the conditions under which the *cyclin D1* promoter is methylated *in vivo*. To this end, we have begun a series of bisulphite sequencing experiments using both breast tumor (MCF7) and non-tumor (MCF12A) cell lines. Genomic DNA was isolated from both of these cell lines under both serum-starvation and serum-stimulation conditions. However, since the *cyclin D1* promoter region is G/C rich, we were unable to successfully PCR amplify the *cyclin D1* promoter region even after extensive troubleshooting. Since it is important to know when the *cyclin D1* promoter is methylated, our next step is to utilize a PCR amplification kit specific for G/C rich DNA. This will allow this aspect of Aim 2.1 to be successfully accomplished.

Aim 2.2: Examine the effects of Kaiso misexpression on *cyclin D1* expression. Significant progress within Aim 2.2 could not be accomplished due to the necessity of creating a stable Kaiso overexpression breast tumor cell line. As outlined in Aim 1.2, we have created the MCF7 Kaiso overexpression cell line therefore progress within this goal of Specific Aim 2 can now be accomplished. The next step in these experiments will be to perform western blot analysis of Kaiso and Cyclin D1 using both the stable MCF7 overexpression cell line and a Kaiso-depletion cell line (previously created in the Daniel lab).

Outcome:

1. **Determined that Kaiso does not bind to the +1050 KBS in either a methylation-dependent or independent manner.**
2. **Creation of a human Kaiso zinc-finger mutant overexpressing construct in the pGEX-5X-1 vector.**
3. **Identified ten CpG dinucleotide pairs that are recognized and bound by Kaiso in the *cyclin D1* promoter.**
4. **Identification of two CpG dinucleotide pairs in the *cyclin D1* promoter that are potentially physiologically relevant recognition sites of Kaiso-mediated repression.**

Specific Aim 3: Analyze catenin function in transcriptional regulation of cyclin D1.

Aim 3.1: Assess role of p120^{ctn} in modulating Kaiso-mediated transcriptional regulation of *cyclin D1*. I was successfully able to validate the findings of the previous P.I. Ms. Abena Otchere that p120 activates the *cyclin D1* promoter in luciferase-based assays. Our next objective is to determine how p120 affects Kaiso's regulation of Cyclin D1 protein levels. This will be done using the newly created Kaiso overexpression stable cell line in conjunction with transient transfection methodologies using p120 expression vectors.

Aim 3.2: Determine if p120^{ctn}/Kaiso antagonize or synergize with β-catenin/TCF regulation of *cyclin D1.* As outlined in the previous annual report written by the former P.I. Ms. Abena Otchere we found that Kaiso inhibited β-catenin-mediated activation of the *cyclin D1* promoter while p120 enhanced this activation. Next, to determine the effect of p120 on Kaiso's ability to inhibit β-catenin-mediated activation of the *cyclin D1* promoter, I performed promoter-luciferase assays using the 1748CD1 *cyclin D1* promoter- reporter plasmid, Kaiso, p120 and β-catenin overexpression vectors in HeLa cells. Due to inefficient transfection and overexpression (as determined by western blot) of the β-catenin overexpression plasmid the results obtained were inconclusive and need to be repeated. I am currently in the process of optimizing transfection efficiency of the β-catenin overexpression plasmid and will repeat the luciferase assay. We will also test the effects that this β-catenin overexpression plasmid has on Cyclin D1 protein levels though western blot analyses of cell lysates.

Outcome:

1. **Validation that p120 activates the *cyclin D1* promoter**

KEY RESEARCH ACCOMPLISHMENTS

1. **Identified methyl-CpG sites in the *cyclin D1* promoter and characterized Kaiso's methylation-dependent binding to the *cyclin D1* promoter.**
2. **Determined that Kaiso does not bind to the +1050 KBS site located within intron 1 of the *cyclin D1* promoter.**
3. **Generation of a breast tumor (MCF7) stable Kaiso-overexpression cell line.**

REPORTABLE OUTCOMES

Journal Articles:

1. **Manuscript in preparation.** Michelle I. Anstey, Abena A. Otchere, Kevin F. Kelly, Monica Graham and Juliet M. Daniel. *Kaiso is a transcriptional repressor of the cyclin D1 gene.*

Abstracts (presenters*):

1. Juliet M. Daniel, Kevin F. Kelly*, **Michelle I. Anstey***, Mai Almardini, Nickett S. Donaldson, and Monica Graham. *Crosstalk between p120ctn/Kaiso and β-catenin/TCF signaling in human cancer*. Hamilton's Inaugural Health Research in the City. (January 2007). Hamilton, Ontario. (**Winner, Best Poster in the Cancer Division**).

2. **Michelle I. Anstey***, Abena A. Otchere, Kevin F. Kelly and Juliet M. Daniel. *Regulation of the Wnt-target genes cyclin D1 and matrilysin by Kaiso/p120^{ctn} signaling*. International Women's Day Research Conference. (March 2007). Hamilton, Ontario.

Degrees Obtained that are supported by this award:

1. The former P.I. Ms. Abena Otchere received her M. Sc. degree in June 2006 after 1 year support by this award.

Development of cell lines:

1. Creation of a breast tumor, Kaiso overexpressing stable cell line.

CONCLUSIONS

In the seven months since becoming the principle investigator of this award to characterize *cyclin D1* as a putative Kaiso target gene in the context of breast tumorigenesis, I have had a very rewarding experience. I have created a stable Kaiso-overexpression breast tumor cell line that can be used to complete the final aims of this grant. I have further validated that Kaiso's interacts with the *cyclin D1* promoter in a methylation-specific manner. I found that Kaiso binds to at least eight CpG dinucleotide pair regions in the *cyclin D1* promoter and I identified two CpG dinucleotide pairs (CpG5 and CpG8) that may represent physiologically relevant sites bound by Kaiso. Hence *cyclin D1* is the first putative Kaiso target gene regulated by the bimodal activity of Kaiso.

In the remaining year of this grant our objectives are to complete the remaining aims outlined in the grant. The analysis and comparison of Kaiso and Cyclin D1 protein levels in breast tumor tissue samples is currently underway (Aim 1.1). Studies on Kaiso misexpression effects on cell proliferation and transformation (Aim 1.3) and completion of our analysis of Kaiso misexpression effects on Cyclin D1 protein levels can now be accomplished using the new Kaiso overexpression cell line (Aim 2.2). Finally our assessment of p120/Kaiso modulation of β-catenin/TCF activation of *cyclin D1* may also be finalized (Aim 3). This final year of the grant promises to be a productive and exciting one as I examine the effects of Kaiso on breast tumorigenesis.

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APPENDIX

Figure 1: Kaiso does not bind the *cyclinD1* promoter +1050 KBS site. An oligomer spanning the +1050 KBS site located within the first intron of the *cyclinD1* gene. This was either methylated or not then radiolabelled and incubated in a binding reaction with 250 ng of various GST-hKaiso constructs. The Kaiso proteins did not bind to either the methylated or non-methylated +1050 KBS site.

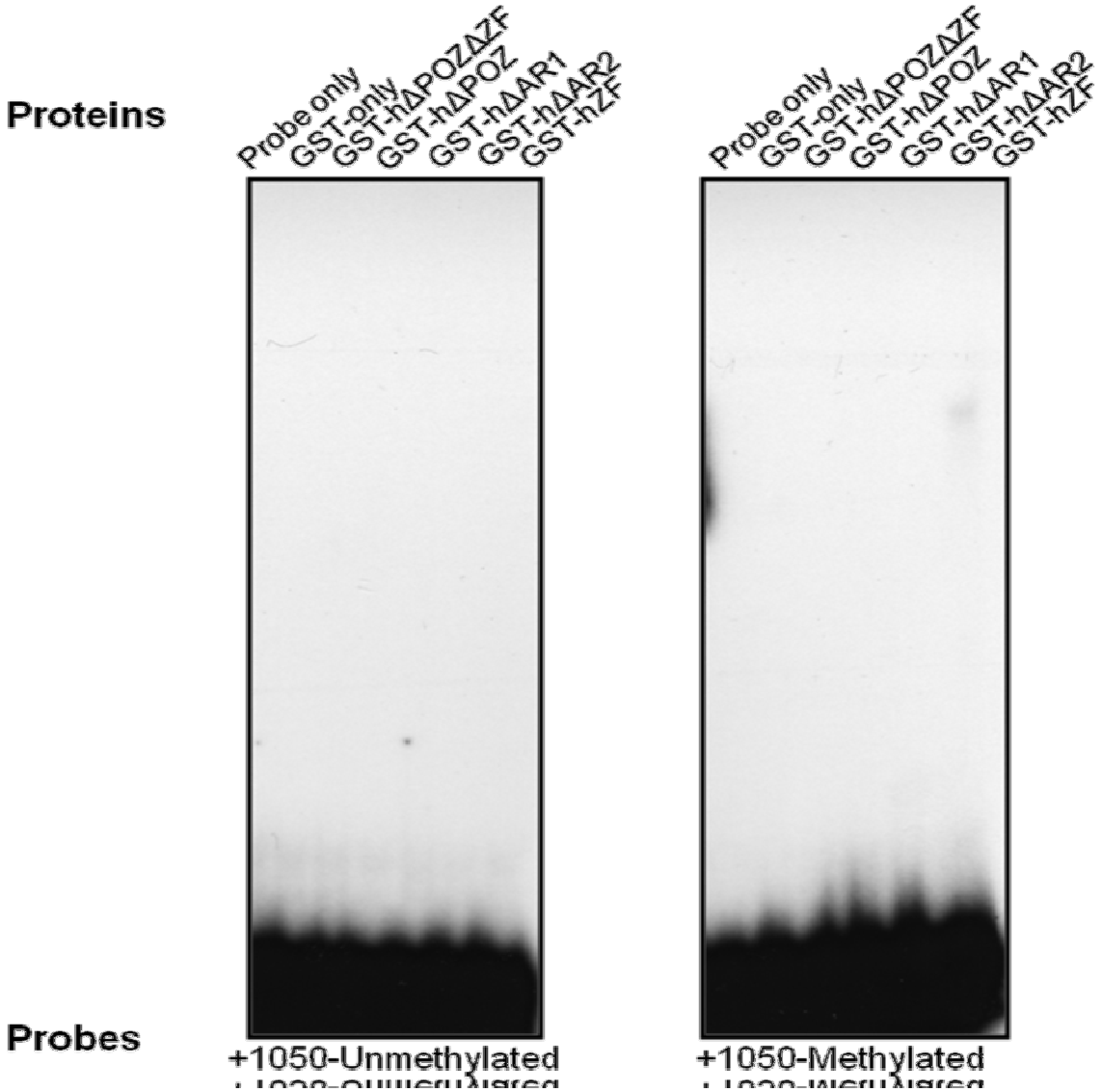
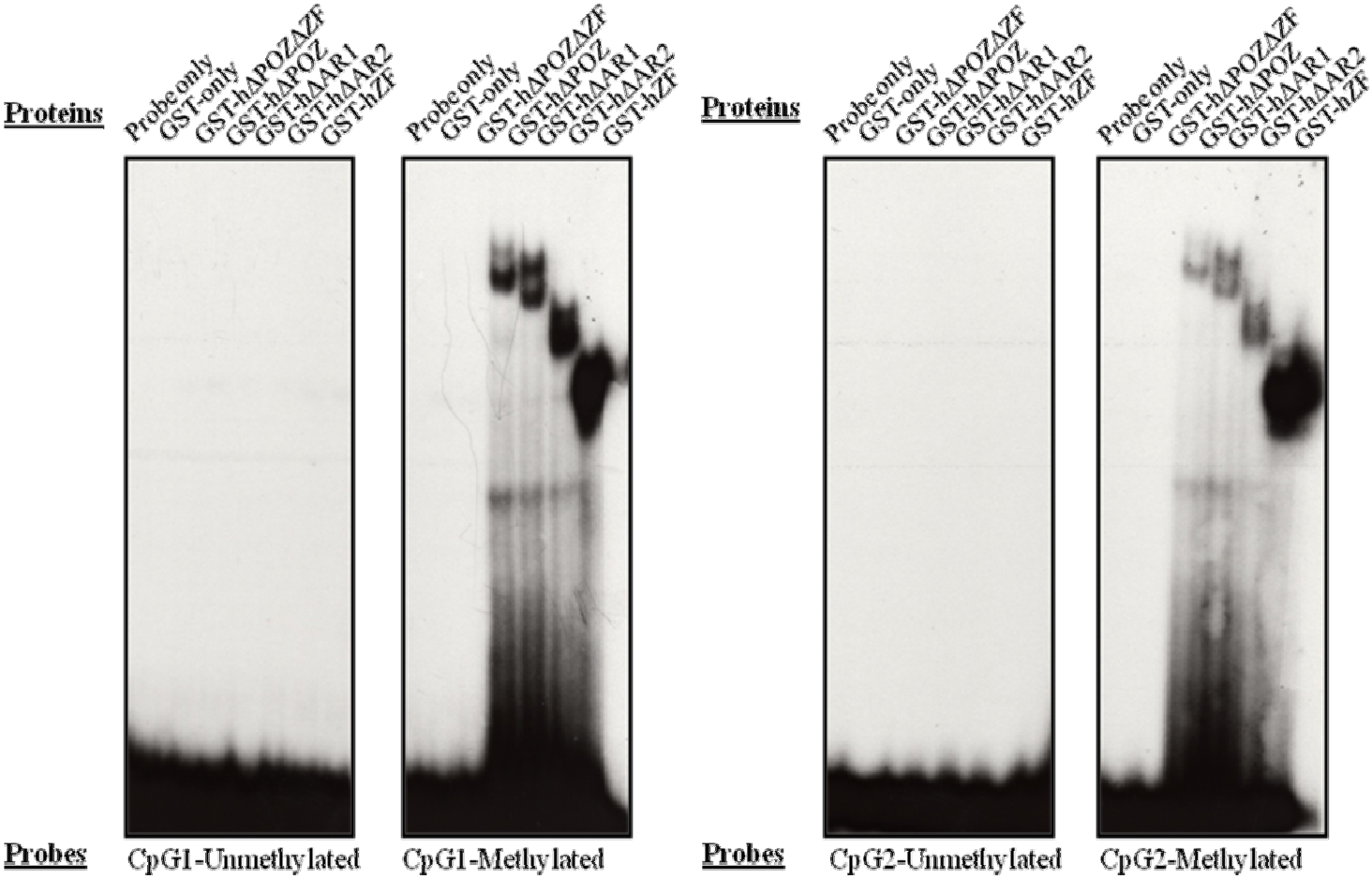
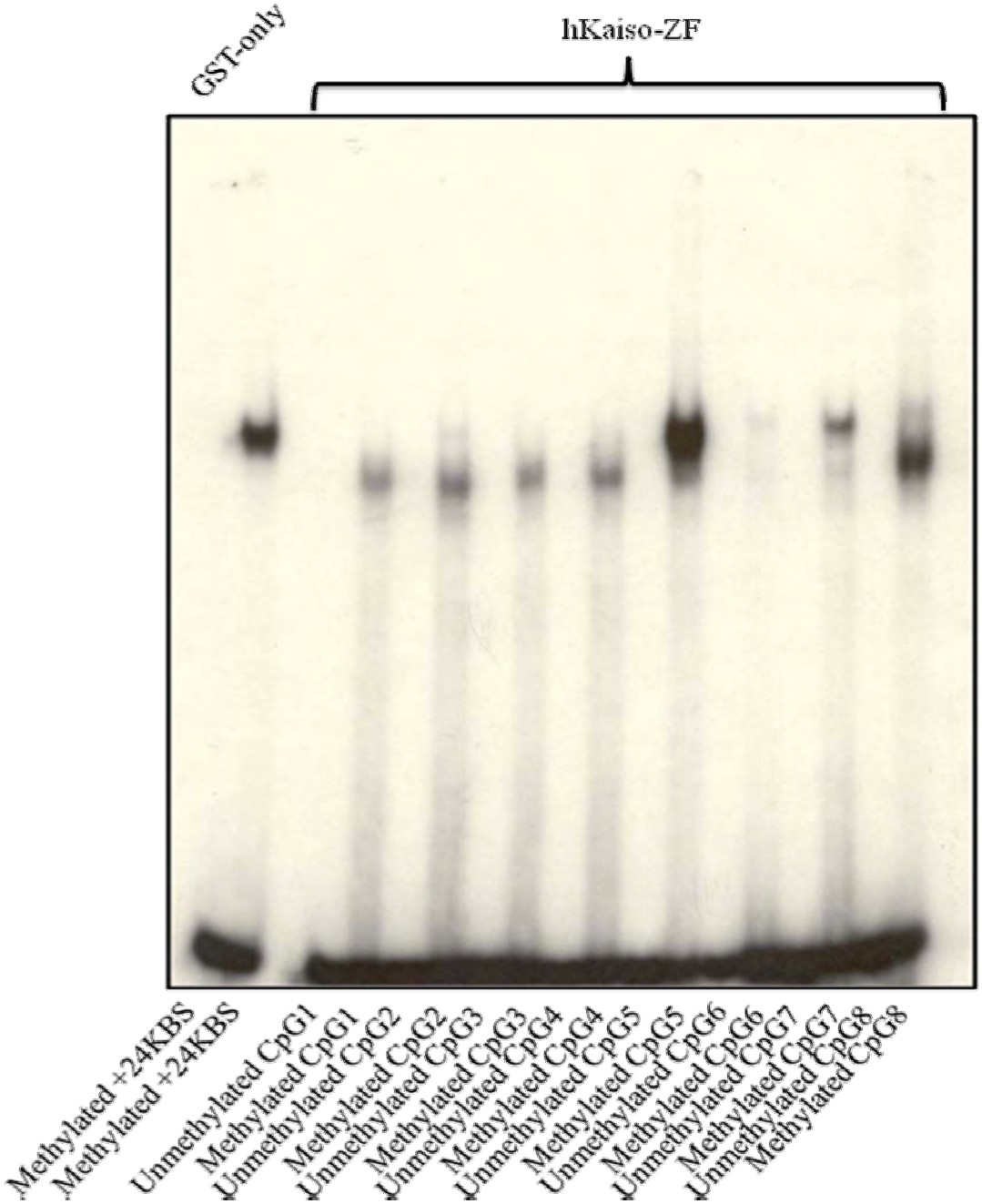


Figure 2: Kaiso binds to all methylated CpG oligomers in *cyclinD1* promoter. *CyclinD1*-promoter derived oligos (CpG1 to CpG8) were methylated *in vitro*, radiolabelled & incubated with GST-Kaiso proteins. Kaiso bound to methylated probes but not to unmethylated probes. Shown in the below diagram are representative autoradiograms for oligos CpG1 to CpG2, and similar results were obtained for CpG3 to CpG8.

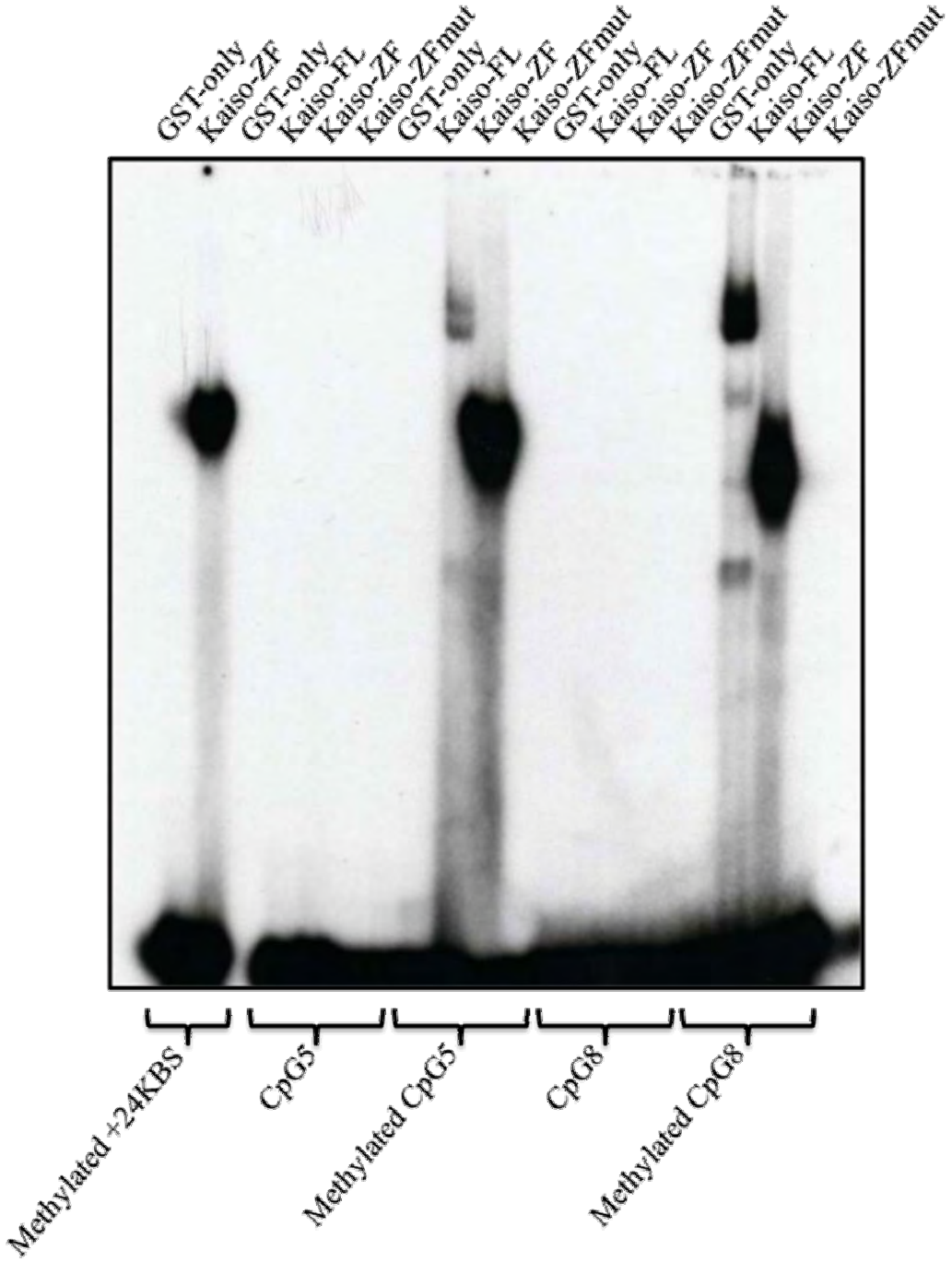


<u>Figure 3</u>: Kaiso binds the methylated CpG5 and CpG8 sites with the highest affinity. *CyclinD1*-promoter derived oligos (CpG1 to CpG8) were methylated *in vitro*, radiolabelled & incubated with GST-hKaiso ZF proteins. Kaiso bound to methylated probes but not to unmethylated probes. Shown in the above diagram are representative autoradiograms for oligos CpG1 to CpG2, and similar results were obtained for CpG3 to CpG8.



<u>Figure 4</u>: Full length Kaiso binds CpG5 and 8 but not CpG3 and CpG6. (A) Oligos CpG5 and CpG8 were methylated *in vitro*, radiolabelled & incunbated with full length GST-hKaiso, GST-hKaiso ZF and GST-hKaiso ZF mutant. Full length Kaiso bound to CpG5 and CpG8 but not to the ZF mutant protein. **(B)** Oligos CpG3 and CpG6 were methylated *in vitro*, radiolabelled & incunbated with full length GST-hKaiso, GST-hKaiso ZF and GST-hKaiso ZF mutant. Full length Kaiso and the ZF mutant did not bind to CpG3 and CpG6.

(A)



(B)

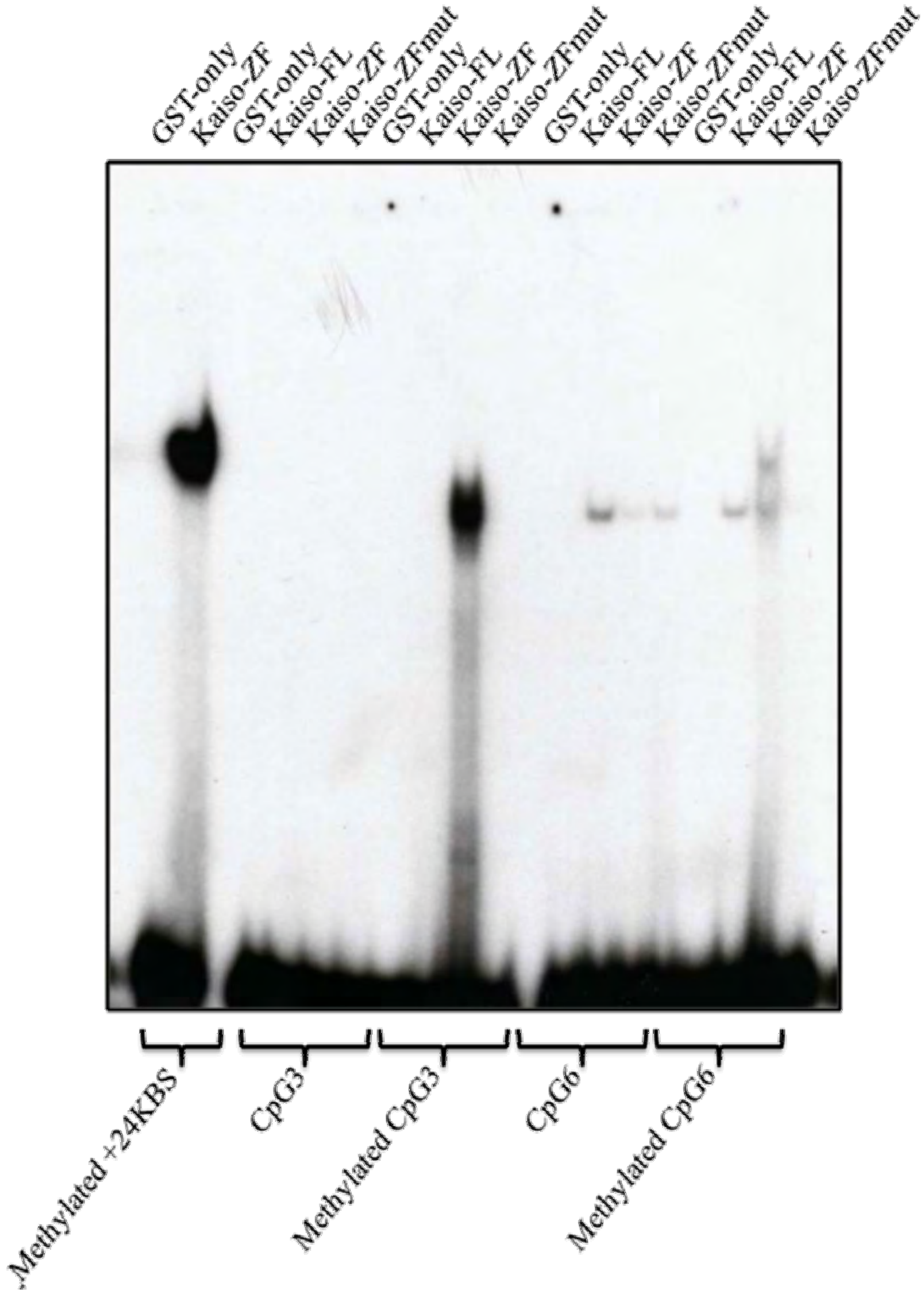

GST-only
Kaiso-ZF
GST-only
Kaiso-FL
Kaiso-ZF
Kaiso-ZFmut
GST-only
Kaiso-FL
Kaiso-ZF
Kaiso-ZFmut
GST-only
Kaiso-FL
Kaiso-ZF
Kaiso-ZFmut
GST-only
Kaiso-FL
Kaiso-ZF
Kaiso-ZFmut
Methylated +24KBS
CpG3
Methylated CpG3
CpG6
Methylated CpG6

Figure 5: *In vitro* methylated 1748CD1 is completely repressed in addition to or absence of exogenous Kaiso. To investigate whether complete methylation of 1748CD1 partial *cyclin D1* promoter reporter construct will affect Kaiso's ability to repress the *cyclin D1* promoter we co-transfected it with Kaiso after methylation by Sss.1 methylase. We observed a complete repression of the *cyclin D1* promoter when methylated in the presence and absence of exogenous Kaiso.

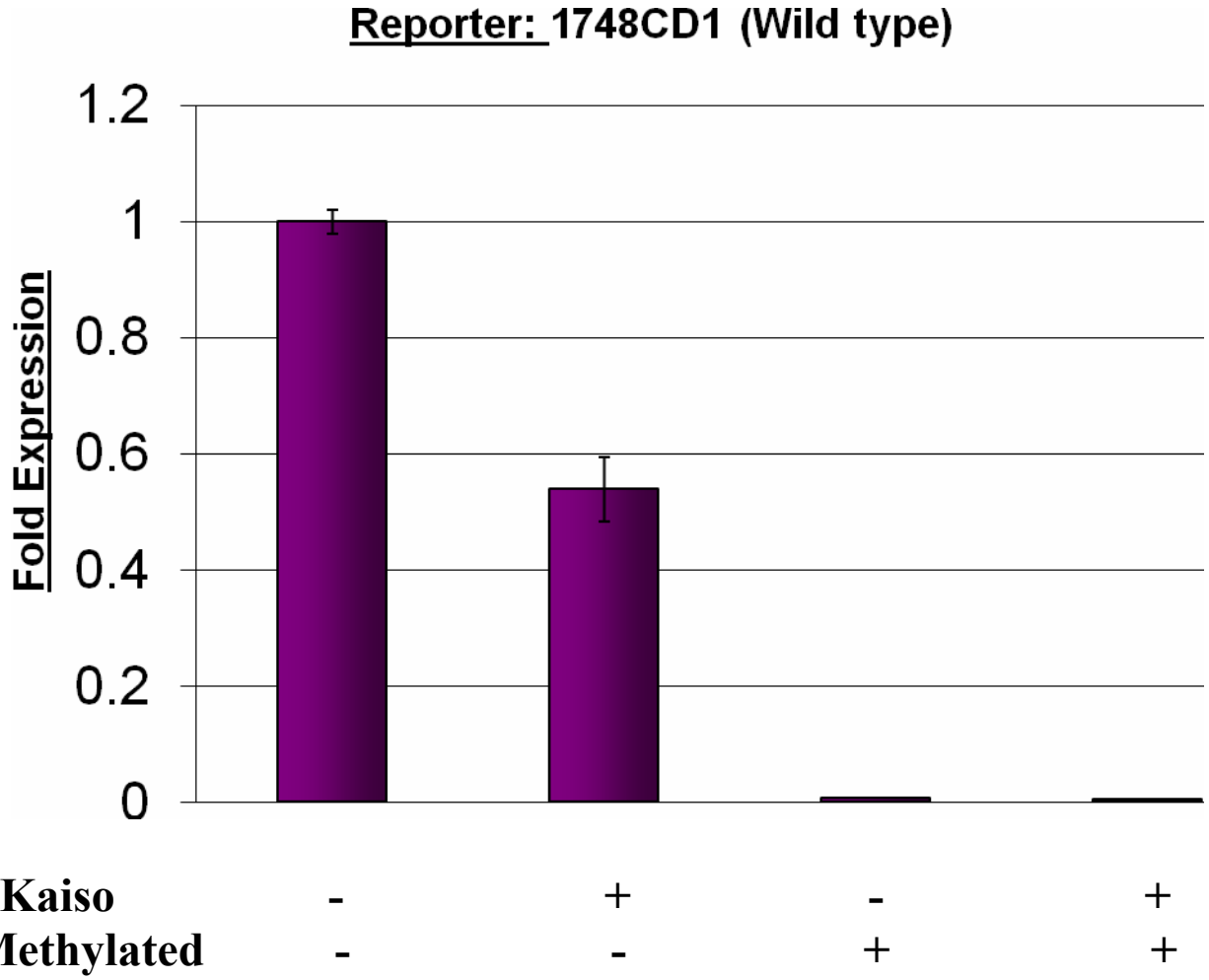


Figure 6: *CyclinD1* promoter has increased repression following 5'azacytidine treatment. To investigate whether a methytransferase inhibitor may effect Kaiso's ability to repress the *cyclin D1* promoter we treated the cells with 2.0μM 5'azacytidine (5'aza) for 48 hours and then co-transfected the 1748CD1 partial *cyclin D1* promoter reporter construct with Kaiso. We observed a slight decreased expression of the *cyclin D1* promoter following treatment both in the presence and absence of exogenous Kaiso.